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Nanocolloidal Gold-Based Immunoassay for the Detection of the *N*-Methylcarbamate Pesticide Carbofuran

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Nanocolloidal gold particles were prepared and labeled to an anti-carbofuran monoclonal antibody (Mab). This conjugate was dispensed on the conjugated pad of a porous glass fiber. Ovalbumin (OVA)−carbofuran and goat anti-mouse IgG were dispensed on the nitrocellulose (NC) membrane and served as the test line and control line, respectively. The carbofuran-containing sample migrated to the NC membrane and reacted with the anti-carbofuran Mab labeled with the colloidal gold. The mixture diffused along the membrane and passed through the OVA−carbofuran in the test line via capillary action. The more analyte present in the sample, the more effectively it will compete with the carbofuran immobilized on the test line for binding to the limited amount of antibody labeled with colloidal gold. An adequate amount of carbofuran could prevent attachment of the colored conjugate to the test line. The presence or absence of a colored band on the test line could indicate a negative or positive result, respectively. When measured to the water sample spiked with carbofuran, this was obtained at or above 0.25 mg/L of carbofuran. The major advantages of the one-step strip test are that the detection time needed was <10 min and all of the reagents are included in the test device.

KEYWORDS: Carbofuran; gold immunochromatographic assay (GICA); colloidal gold particle; pesticide residue

INTRODUCTION

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate), with a trade name of Furadan, is a systemic *N*-methylcarbamate pesticide with predominant contact and stomach action. It is an important insecticide to control soil-dwelling and foliar-feeding insects, spider mites, and nematodes in vegetables, ornamentals, and agronomic crops such as maize, sorghum, alfalfa, peanuts, and potatoes (*1*). Carbofuran is a potent cholinesterase inhibitor [IC_{50} in rats is $(1.2-3.3) \times 10^{-8}$ M] with high acute toxicity to bees, birds, and fish (*2*−*4*). There are different regulations about carbofuran tolerances from different countries all over the world; for instance, the U.S. EPA proposed carbofuran tolerances for residues in raisins, artichokes, and canola of 2.0, 0.4, and 1.0 mg/kg, respectively (*5*). The Malaysian government suggests the maximum residue limits (MRLs) of carbofuran in strawberry, leaf-eaten vegetable, root-eaten vegetable, grape, and banana of 0.5, 0.5, 0.5, 0.4, and 0.1 mg/kg, respectively; the MRL of carbofuran in grain is 0.5 mg/kg in China (*6*). As a result of its widespread and improper use, serious problems have been seen in China. In some farm areas, the concentration of carbofuran residue was found to be far more than the MRL. For example, carbofuran has been found in vegetables, melons, and fruits such as water bamboo (*Zizania latifolia*) and Chinese chive (*Allium tuberosum*) (*7*). Residues of carbofuran may be present in the air, food, and soil as well as in surface water. It may contaminate groundwater through runoff and leaching (*8*).

Determination of pesticide residues is always an important research field. Currently, the methods of detecting carbofuran residues involve improved gas chromatography, high-performance liquid chromatography (HPLC) with postcolumn derivatization, and fluorescence detection (*9*−*11*). However, these methods are either expensive or time-consuming due to the complication of sample preparation and preconcentration. Sophisticated equipment is usually needed, although it is often not available in many analytical laboratories. On the other hand, the methods were not suitable for analyzing a large number of samples. Enzyme-linked immunosorbent assays (ELISAs) for the analysis of the residues of pesticides have been developed rapidly in recent years since the application of immunochemistry to environmental analysis (*12*−*14*). The commercial ELISA kits using polyclonal antibody as the specific immunoreagent have been applied to determine carbofuran in water, soil, meat, and

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liver (*15*). Obtaining high-affinity monoclonal antibodies toward carbofuran has promoted the development of the immunoassay technique dramatically as the monoclonal antibodies (Mabs) have the advantages of unlimited preparation of a single and homogeneous type of antibody (*16*, *17*).

Because colloidal gold has been introduced into immunochemistry, the nanocolloidal gold particles could replace the enzyme to be labeled to antibody (*18*, *19*). When the antibody labeled with colloidal gold particles is combined with the corresponding antigen, the reactant would be colored obviously (*20*−*22*). Thus, a rapid test method, a simple one-step strip, has been developed and applied increasingly in various research fields, especially in immune electrophoresis, molecular biology, biochip, and immunochemistry (*23*−*26*). The first major target of this method was human chorionic gonadotropin (HCG) when it was applied in the detection of pregnancy. There are also assays commercially available applied in low molecular mass analyses, for example, detection of drug abuse, prediction of steroid-based ovulation, and determination of progesterone in milk according to competitive immunoassay protocols. So far, this method has been mainly intended for human diagnostics. There is a lack of studies on pesticide residue determination by gold immunochromatograph assay (GICA).

With the improvement of living standards, people pay more attention to food safety as well as remedy. There is an urgent need to develop a one-step assay for pesticide residues. Several groups have developed both rapid and sensitive tests for pesticide residues, which are mainly enzyme immunoassays (*17*, *27*). However, this kind of determination requires some expensive equipment, for example, a microplate reader, a plate washer, and a benchtop orbital motion shaker. Compared with enzyme immunoassay (EIA), determination by GICA aforementioned is a better method, which might be accomplished rapidly in one step with the similar effect without costly instruments.

The goal of this study was to develop a rapid, simple, and qualitative detection method that was based on the principle of the high specificity of immunochemical reactions between antigens and antibodies. In this paper we describe the development of a one-step assay for the detection of carbofuran by means of GICA.

MATERIALS AND METHODS

Chemicals, Immunoreagents, Materials, and Instruments. Carbofuran standard (≥99.9%) was obtained from the Institute of Environmental Protection, Ministry of Agriculture (Tianjin, China). The stock solution (20 mg/L) of carbofuran standard was prepared with *N*,*N*-dimethylformamide (DMF) and stored in an amber flask at −20 °C until it was used to prepare fresh working solutions. Anti-carbofuran Mabs were supplied kindly by Prof. Angel Montoya. The Mabs, identified as IgG2a, were purified with protein A affinity column (Pharmacia, Uppsala, Sweden). The concentration of protein in solution was determined by using the bicinchoninic acid (BCA) protein assay. Ovalbumin−carbofuran was synthesized when ovalbumin (OVA) was attached covalently to the carbofuran hapten, which was synthesized previously by a series of chemical reaction processes referring to the Abad et al. method (*16*). Bovine serum albumin (BSA), OVA, polyethylene glycol (PEG MW = 20000), and gold chloride were purchased from Sigma (Shanghai, China). Goat anti-mouse IgG was obtained from Sino-American Biotechnology Co. (Shanghai, China). Nitrocellulose (NC) membrane (pore size = 5 μm) and glass fiber membrane were provided by Schleicher & Schuell Co. (Dassel, Germany).

The BioDot system consisted of two BioJets Quanti 3000 and one Airjet Quanti 3000 attached on a BioDot XYZ-3000 (Irvine, CA) dispensing platform. The guillotine cutter (model CM 4000) was supplied by BioDot (Irvine, CA). The centrifuge (Heraeus multifuge 3 S-R) was purchased from Kendro Laboratory Products GmbH (Hamburg, Germany). The UV-2401 (pc) s was from Shimadzu Corp. (Tokyo, Japan). The transmission electron microscope (JEM-100CX II) was from JEOL (Tokyo, Japan).

Preparation of Colloidal Gold. Nanometer colloidal gold was prepared by reducing gold chloride with sodium citrate according to the procedure described by Frens (*28*). The procedures for preparing colloidal gold suspension were as follows: About 100 mL of 0.01% m/v $AuCl_3 \cdot HCl \cdot 4H_2O$ was boiled first, and then a solution of 1% trisodium citrate was added under constant stirring. After the color of the solution had changed, in <2 min, it was boiled for another 15 min. After cooling, deionized water was added to the initial volume. The diameters of particles were checked with a transmission electron microscope. The obtained colloidal gold suspensions could be stored at 4 °C for several months supplemented with 0.05% (m/v) of sodium azide.

Gold Labeling of Anti-carbofuran Mab. The mouse anti-carbofuran Mab was labeled with colloidal gold particles according to the Frens method (*28*). Dispersion of gold particles was noticed through heating of a solution containing $AuCl_3 \cdot HCl \cdot 4H_2O$ and trisodium citrate. After cooling, the pH was adjusted to 8.2, which is the p*I* of the Mab (IgG) with 0.2 M K_2CO_3. With a simultaneous fast stirring, 4 mL of purified anti-carbofuran Mab (0.1 mg/mL) was added to 96 mL of dispersed gold solution with a final concentration of 4 mg/L.

After incubation at room temperature for 15 min, PEG (20000, 1% as the final concentration) was added and stirring was continued for another 15 min. The mixture was centrifuged for 30 min at 8000 rpm with a Heraeus centrifuge (multifuge 3 S-R) (Hamburg, Germany). After removal of the supernatant, the precipitate of the gold-labeled antibodies was dissolved in phosphate-buffered saline (PBS, pH 8.2) and stored at 4 °C.

Immobilization of Reagents. BioJet (XYZ3000) (Irvine, CA) was used to dispense two lines on the NC membrane strips (25 × 300 mm). The OVA−carbofuran at a concentration of 0.5 mg/mL was dispensed on the low part as the test line, and the dispensed volume was 1 μL per 1 mm line. The goat anti-mouse IgG at a concentration of 1 mg/mL was dispensed on the upper part as the control line, and the dispensed volume was also 1 μL per 1 mm line. After dispensation, it was dried for 2 h at 37 °C; the NC membrane was blocked with 1% BSA for 30 min, washed three times with PBS, and then dried again at 37 °C for 3 h. The strips were stored under dry conditions (desiccator) at room temperature until use.

Gold-labeled anti-carbofuran Mab (detector reagent) was dispensed onto a glass fiber filter with Airjet (XYZ3000) (Irvine, CA) and then dried at 37 °C for 3 h. The optimal concentration of the dispensed mixture was selected as the one with an OD value of 10 at 530 nm, the dispensed volume of this compound can also be calculated as 1 μL for a 1 mm line.

Assemblage of the Strip. The different parts of the test strip were pasted onto a plastic backing with adhesive. The NC membrane on which the OVA−carbofuran and goat anti-mouse IgG were previously blotted was pasted on the center of the backing plate. The conjugated pad on which the gold-labeled Mab was dispensed previously was pasted on the plate by over-crossing 2 mm with the NC membrane. The sample pad (glass fiber) was also pasted on it by over-crossing 4 mm with the conjugated pad. The absorbent pad was pasted on the other side of the plate. The whole assembled plate was cut lengthways and divided into strips with a guillotine cutter (5 mm × 60 mm) as shown in **Figure 1**.

Standard Working Solution of Carbofuran and Test Procedure. The standard of sample pesticides was prepared in PBS (8.03 mM $NaHPO_4$, 1.97 mM $KHPO_4$, 137 mM NaC1, 2.68 mM KCl, pH 7.4) by serial dilutions, using borosilicate glass tubes (*16*). The blank samples were clean pure water (Milli-Q) used for the control test.

During detection, four drops (~150 μL) of the liquid sample were pipetted into the sample pad, allowing the sample to migrate upward. After 10 min, the testing result was judged.

RESULTS AND DISCUSSION

Selection of Parameter (Size Selecting of Colloidal Gold Particle). Researchers are aware that nanocolloidal gold particles

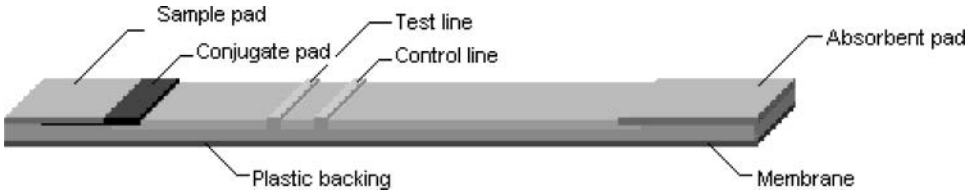

Figure 1. Cross-section of a complete one-step strip test device (5 × 60 mm) showing its components. The plastic backing served as the bottom of the test strip. The NC membrane on which the OVA–carbofuran and goat anti-mouse IgG were previously blotted served as test line, and the control line was pasted onto the center of the backing plate. The conjugated pad on which the gold-labeled Mab was dispensed previously was pasted on the plate by over-crossing 2 mm with the NC membrane. Sample pad and absorbent pad were pasted on the two sides.

Table 1. Changes of Diameter Size and Color of Formed Colloidal Gold in the Process of Colloidal Gold Preparation with Different Amounts of Trisodium Citrate

diameter siz of colloidal gold particles (nm)	amount of trisodium citrate (1%) added in a 100 mL gold chloride solution (0.01%) (mL)	color of colloidal gold particles
15	2	reddish
25	1.6	salmon pink
40	1	dark red
55	0.8	purple-red
75	0.6	purple
100	0.46	purple-gray
150	0.352	blue

have been gradually applied in immunoanalysis, biosensor, bioidentification, gene therapy, and DNA computation (*29*). Because immunoglobulins could be labeled with nanocolloidal gold particles instead of enzyme, the substrate is therefore not needed in the reacting system. The strength of color showing is closely related to the size and quality of the colloidal gold particles. The size of the colloidal gold particles is directly dependent on the amount of trisodium citrate used in its preparation process. Finding the optimal concentration of trisodium citrate in solution is a crucial task that can influence the formation of particular cores. After the reducing agent is added, aggregation of gold atoms subsequently occurs, in a process called nucleation. The number of nuclei formed initially determines the amount of particles finally grown in solution. At a fixed concentration of tetrachloroauric acid in solution, as the concentration of the reducing agent is increased, the number of formed nuclei grows larger. The more nuclei there are, the smaller the gold particles produced. Therefore, when all other preparation conditions including procedures and reagents were kept constant, changing the amount of trisodium citrate could result in different colloidal gold particles with different colors. In our study, seven different types of colloidal gold particles were obtained. The results are summarized in **Table 1**. When the amount of trisodium citrate (1%) added in 100 mL (0.01%) of gold chloride solution was >1.6 mL, the formed colloidal gold particles were reddish and small with a diameter of <40 nm, which means a low reactant concentration results in low rates. Those small reddish particles were found to be too small to indicate a clear and bright color. When the amount of trisodium citrate added was <0.8 mL, the color of the obtained colloidal gold particles was between purple and blue. The diameter of the particles was >55 nm. Those large size colloidal gold particles were found to be unstable. After the particles had been kept in steady state for 1 week, self-coagulation occurred. This indicates that it might be difficult to keep the large size colloidal gold particles for a long time. Besides, too high a concentration might yield other problems. The desired product might not form in sufficient quantity, and the reaction might produce a large amount of byproducts. Only

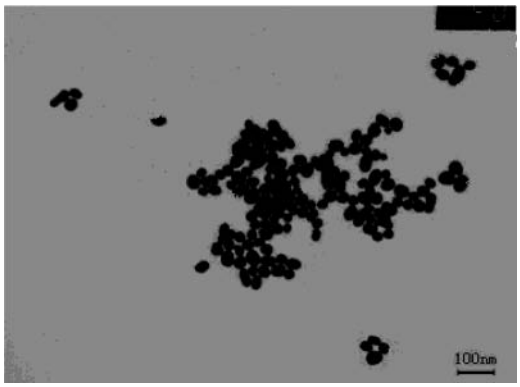

Figure 2. Size of valid colloidal gold particles observed by transmission electron microscope (×120000). The diameter of those particles was ~40 nm. (Figure is reproduced here at 75% of its original size.)

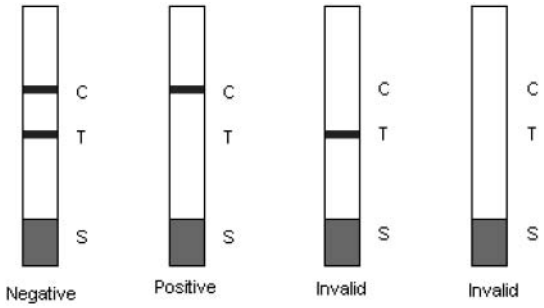

Figure 3. Illustration of immunochromatographic test results. The positive result is judged by the appearance of only a single color line in the control region. The negative result is judged by the appearance of two lines in the control and test regions. The test is invalid if no line is present in the control region.

when the amount of trisodium citrate added was 1.0 mL did the formed colloidal gold particles show a dark red color with a diameter of 40 nm. The conjugates combining with immunoglobulins were found to be stable. The indicating color was obvious and easy to distinguish. The immunoreaction compound moving on the NC membrane was fast. The reaction could be completed and remained stable. Therefore, the 40 nm colloidal gold particles were selected in our study. The highest absorbance of the selected particles was found at 535 nm. Its morphology is shown in **Figure 2**.

OVA−carbofuran (0.5 mg/mL) was dispensed on the NC membrane as the test line. To determine the appropriate concentration of OVA−carbofuran coated on the membrane, the OVA−carbofuran was prepared in PBS (pH 7.4) by serial dilutions ranging from 0.05 to 1.0 mg/mL. We found that the higher the OVA−carbofuran concentration was, the greater was the amount of carbofuran needed in the sample to compete with the particles. However, at <0.5 mg/ml, the stained capture line is too faint to observe with the eye.

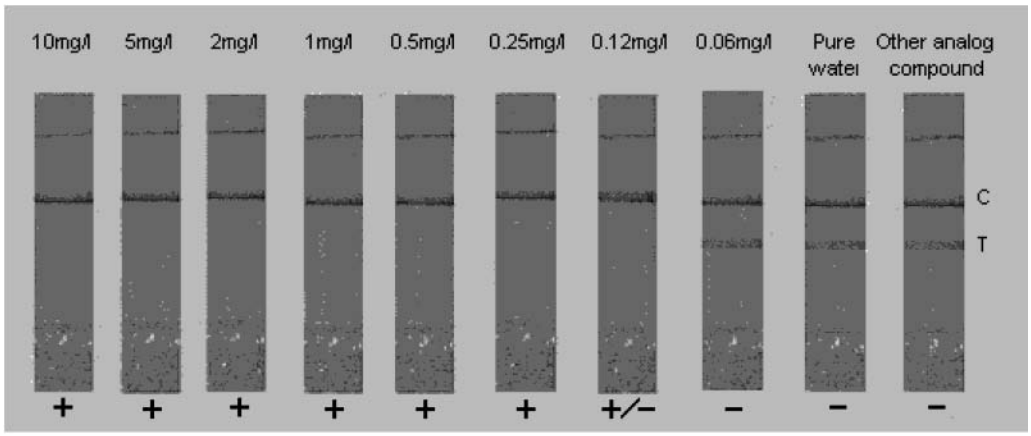

Figure 4. Immunochromatographic detection of carbofuran. A series of dilutions (0.06–10 mg/L) of carbofuran were prepared. Details of the preparation of the test device and assay procedures are described in the text. A concentration of 0.12 mg/L of carbofuran was found to cause a considerable decrease with a faint color band at the test line. In the absence of carbofuran (pure water only) an obvious band displayed on the test line. On the control strip for other analogue compounds (each compound is not shown in this figure), an obvious band was also revealed in the test line even though these compounds were spiked at a high level of 100 ppm.

The different buffers including 0.01 M PBS (pH 7.4), 0.1 M PBS (pH 7.4), carbonate buffer (pH 9.6), and borate buffer (pH 6.0) serving for gold-labeled antibody were compared. We found that the pH has a significant influence on the system, and the 0.01 M PBS (pH7.4) is the best for the assay sensitivity.

Result Judgment. The test result was judged as positive or negative visually as illustrated in **Figure 3**. The goat anti-mouse IgG and the OVA−carbofuran are separately stripped onto the control region (c) and test region (t). The Mab labeled with colloidal gold particles could react with carbofuran (competition inhibition) so that a positive result could be judged on the basis of the appearance of a single dark red line in the control region. A negative result could be judged with the appearance of two colored lines in the control and test regions. The color band must be displayed on the control line based on the design principle, so the test could be considered as invalid if there was no color line present in the control region. The developed method was currently used only in the detection of carbofuran. It should be possible to apply the same principle to the detection of multiple pesticides by combining different conjugates in one NC membrane. The different corresponding coating antigen for each pesticide could be fixed at different points on the test line. Thus, appearance of different indication color lines would illustrate the presence of different pesticides in a tested sample.

Detection Limit. The detection limit is defined as the amount of carbofuran in the solution that just causes a complete invisibility of the test line. A series of dilutions of standard carbofuran (0.1−10 mg/L) were assayed by test strips as shown in **Figure 4**. The testing results were recorded 10 min after the reaction started. Two lines showing on the membrane meant that the carbofuran concentration was below the detection limit. When only one color line was shown in the control zone, the carbofuran concentration was above the detection limit.

To ensure the accuracy of the detection results, the detection limit was estimated as 0.25 mg/L because the concentration of 0.12 mg/L of carbofuran was found to cause a considerable decrease in the assay signal. As the detection limit is ∼0.08 mg/L by the ELISA as developed by Montoya, the sensitivity of our one-step strip method was lower (*16*). However, it is possible to improve the sensitivity by adjusting the concerned factors, which affect the assay system. Shyu et al. reported that the detection limit of ricin could be improved from 50 ng/mL to 100 pg/mL if a reacted test strip was rinsed with a PBS solution containing 0.1% (w/v) Tween 20 and distilled water and soaked into a silver enhancer, which is almost as sensitive as the amplified enzyme immunoassay (*30*). It might be possible to significantly improve the sensitivity of our method by means of silver enhancement.

Specificity. The specificity of the method was evaluated in comparison to other analogue compounds. Carbaryl, methiocarb, propoxur, bendiocarb, aldicarb, methomyl, carbofuran-phenol, carbofuran-hydroxy, carbofuran-keto, carbosulfan, benfuracarb, and furathiocarb were dissolved in appropriate solvents, respectively, and then the test strip was dipped into each compound for 10 s, taken out, and laid on a flat table; after 10 min, the result was judged with the human eye. As shown in **Figure 4**, a clear band was observed in the test line on the control test strip even though these compounds were spiked at a high level of 100 ppm. Each analogue compound was found not cross-reacted when tested at concentrations up to 100 ppm. The probability of cross-reaction was <0.25. This indicates that the developed technique had a high specificity toward carbofuran. Because this method is qualitative, details could not be observed when the specificity was evaluated by comparison with other analogue compounds, which is different with the ELISA method.

Reliability. The results of this study demonstrate that the one-step strip test is suitable for the rapid determination of carbofuran residue. This method provides only a preliminary, semiquantifying result, with a simple positive/negative result. The result could be used to judge whether the pesticide concentration remaining in the sample was higher than the detection limit or not. If one needs to know the real concentration of the pesticide, a conventional and standard analysis is necessary. This is different from ELISA, which might provide completely quantified data of pesticide concentration.

The reliability of the assay was determined by carrying out the test with the water sample spiked with standard carbofuran. The four concentrations of 0 mg/L, at −25% from cutoff (0.187 mg/L), at the cutoff (0.25 mg/L), and at +25% from cutoff (0.312 mg/L) were tested to check the accuracy of the test device. Fifty tests were run at 0 mg/L, 50 at 0.187 mg/L, 50 at 0.25 mg/L, and 50 at 0.317 mg/L, for a total of 200 tests. The concentration of carbofuran in the treated sample was detected with our method and the ELISA method simultaneously. The results obtained in the two procedures were compared, as shown in **Table 2**, indicating that the two methods highly agree with one another.

Table 2. Reliability of the Test Strip Compared to ELISA[a]

	ELISA	
GICA	>250 μg/mL	<250 μg/mL
>250 μg/mL	100	0
<250 μg/mL	0	100
total	100	100

[a] Comparison of the results between gold immunochromatagraph assay (GICA) and ELISA. The four concentrations of 0 mg/L, 0.187 mg/L (at −25% from cutoff), 0.25 mg/L (at the cutoff), and 0.312 mg/L (at +25% from cutoff) were tested to check the reliability of the device. Fifty tests for each concentration, a total of 200 tests, were made. The overall agreement is 100% (200/200).

Compared with other methods, the one-step strip test has many advantages. First, it is a simple method. There is no requirement for instruments. Human eyes can easily judge the test results. Unlike ELISA, the method does not use any enzyme. There is no need for washing samples repeatedly and adding different reagents, so the whole detection time is very short. Second, the risk to the operators and the environment is decreased during the operation. Third, the colloidal gold is very stable in dry conditions, and the one-step test strips can be stored for about a year in ambient conditions. Temperature has less influence on this testing method compared with ELISA method. We also foresee the appearance of false positives and false negatives during application because of incorrect operation. This method is considered to have a distinct advantage as a preliminary screening method prior to precise detection in a laboratory.

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